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LOW TEMPERATURE SCANNING ELECTRON MICROSCOPY: ADVANTAGES AND APPLICATIONS

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Abstract

Cryo-preparation of specimens for scanning electron microscopy can be completed within a few minutes. Chemical fixation and contact with solvents is avoided, levels of specimen hydration are maintained, low melting-point materials are stabilized, volume changes are minimized and internal structure can be revealed by freeze-fracture. Elements are not lost or substantially relocated prior to X-ray microanalysis and specimen luminescence is enhanced. The displacement of internal structure and material subject to X-ray microanalysis by the growth of ice crystals in hydrated samples can be minimized by adopting fast freezing methods designed to limit ice crystal growth. The technique enables a wide range of industrial and biological materials to be examined rapidly and free from artifacts commonly associated with more conventional preparation methods.

KEY WORDS: Cryo-scanning electron microscopy, advantages, applications.

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Introduction

Low temperature stages for scanning electron microscopy were developed initially to avoid the water loss from hydrated specimens which occurs at room temperature in the high vacuum of the microscope. More recently cryo-preservation has been used to exploit additional advantages which the technique provides (Beckett & Read, 1986; Heathcock, 1985; O'Niel et al, 1987; Sargent, 1983, 1986b; Lott & Kerr, 1984). The purpose of this paper is two-fold. It will discuss the specific advantages which cryo-preservation offers the scanning electron microscopist and, by way of illustration, it will demonstrate the wide range of materials whose examination is being facilitated by application of the technique.

Specimen Preparation

The examination of a specimen held at low temperature in the scanning electron microscope is but the final stage in a procedure which begins with its initial cooling. Subsequent transfer is made under vacuum to avoid frost contamination, and specimen manipulation such as freeze-fracture must be accomplished at low temperature. Means must be provided to warm the specimen to a given temperature if surface etching by water sublimation is required. Additionally, if coating is necessary, provision must be made to maintain the specimen at a low temperature during sputtering or evaporation of a metal or carbon.

The theory and practice of rapid specimen cooling are well described in recent publications (Bald, 1984, 1985; Gilkey & Staehelin, 1986; Robards, 1985; Ryan et al, 1987) and will not be dealt with here. If only the surface features of a frozen specimen are to be examined then cooling rates are less critical. However, if the visualization of internal structure revealed by freeze-fracture is the objective it is vital to minimize structural change brought about by ice crystal growth. The presence of cryo-protecting solutes (as components or following infiltration) can substantially reduce ice crystal damage but in their absence some reduction can be obtained by maximizing the rate of specimen cooling. Much effort and expense can be devoted to increasing the rate of heat loss from the specimen but a

point is reached when the rate-limiting step in the cooling process is the speed at which heat is conducted through the specimen from its centre to the surface. At normal pressures and using the most efficient means of heat removal, this limitation restricts the volume of specimen which is free from ice damage to an outer zone which, at best, is unlikely to exceed 15-20 μm in depth (Robards, 1985). Small increases in depth of this zone can be vitally important in transmission electron microscopy but they represent only a small improvement in the internal preservation of the larger bulk specimens typical of those selected for scanning electron microscopy observation. Nevertheless, it is important to anticipate and recognise effects induced by the growth of ice crystals deep within hydrated specimens.

All the specimens illustrated here were cooled by plunging into nitrogen slush at atmospheric pressure. This is an inexpensive and relatively safe cooling medium which has two major advantages over liquid nitrogen: it avoids the formation of an insulating layer of nitrogen gas around the cooling specimen and associated turbulence which can sweep away delicate surface structures is substantially reduced.

The Hexland CT 1000A cryotransfer system includes a nitrogen slushing chamber which interfaces with a cryotransfer device. The specimen is mounted on a holder attached to a rod protruding through the external housing of the transfer device. After plunging the mounted specimen into nitrogen slush within the chamber, the transfer device housing is located and sealed onto the slushing chamber and the system repumped. At low pressure the specimen is then withdrawn into an inner tube within the transfer device where it is sealed by an externally operated door. On admitting dry nitrogen gas to the slushing chamber, the transfer device can be detached carrying within it the cooled specimen sealed in vacuo. The transfer device interfaces with an evacuated preparation chamber attached to the scanning electron microscope specimen chamber (Figure 1). Within the preparation chamber a stage cooled to -180°C supports the specimen during freeze-fracture, cold honing, sputtering or evaporative coating. This chamber is compact and fully integrated with the scanning electron microscope yet is isolated from the microscope while the gate valve which connects it with the specimen chamber remains closed. Using the rod of the transfer device the specimen can be moved through the open gate valve to the cold stage of the microscope. This stage is cooled by nitrogen gas carried through flexible tubes from an external heat exchanger immersed in liquid nitrogen. Cooling from ambient to -180°C is commonly accomplished in 3 to 4 minutes. A heater in the cold stage enables the temperature to be raised and maintained at any selected level between -180°C and +40°C. Within the chamber a cooled anti-contaminator plate held at -180°C traps sublimated volatiles. The cold stage can be released rapidly from the substage and stowed on the floor of the specimen chamber, a feature which minimizes the time and effort required to convert the system to the ambient working condition. A typical preparation routine is as follows: 1) Mount specimen on a specimen holder using carbon cement. 2) Plunge specimen into nitrogen slush. 3) Withdraw specimen into transfer device under vacuum. 4) Transfer specimen to preparation chamber at -180°C. 5) Fracture specimen using cold knife. 6) Transfer specimen to misroscope stage at -180°C. 7) Examine fracture face at low kV on microscope monitor. 8) Raise stage temperature to -80°C. 9) Observe fracture face until sufficient etching has occured. 10) Transfer specimen to preparation chamber at -180°C. 11) Admit sufficient dry argon to preparation chamber to allow sputter coating of specimen from gold target, 12) Transfer specimen to microscope stage for examination and photographic recording at -180°C.

Liquid suspensions and emulsions are supported for freezing between two hollow rivets placed end to end. The rivet pair, held with fine forceps, is then plunged rapidly into cryogen, transfered to liquid nitrogen, inserted into a rivet-clamping specimen holder and transfered to the specimen chamber. On the preparation cold stage the upper, unclamped rivet is knocked off the lower, clamped one using the cold knife. This exposes a fractured face of the frozen material within the clamped rivet. It can be sputter coated immediately or first etched on the microscope cold stage.

This method of sample preparation avoids the presence of a large slowly-cooling sample holder during quenching and maximizes the depth of the surface layer in which ice crystal size is negligible. Similarly the rate of cooling of bulk specimens can be improved by quenching free from a specimen holder and transfering them under liquid nitrogen to a holder which incorporates a specimen support vice.

Advantages and Applications of Cryo-preservation

The advantages associated with low temperature scanning electron microscopy may be summarised as follows: i) Low melting-point materials are stabilized. ii) Specimens remain fully hydrated. iii) Chemical fixation is avoided. iv) Specimens are not immersed in solvents. v) Volume changes are minimized. vi) Internal structure can be revealed by freeze-fracture. vii) Elements are not lost or substantially moved prior to X-ray analysis. viii) Motile specimens are arrested. ix) Specimen preparation is complete within a few minutes. x) Signal levels from luminescent specimens are enhanced.

In considering each of these advantages in detail it must be stressed that their order does not indicate their relative importance to the scanning electron microscopist. Indeed, the speed with which specimens can be prepared is, for many users, the prime advantage which cryo-preservation offers and a major reason for a growing number of laboratories adopting it as their standard method of specimen preparation.

Low Melting-point Materials are Stabilized

The effect of the electron beam in melting or collapsing specimens which are composed of or contain low melting-point materials has been a problem since electron microscopes were introduced. By lowering the temperature of these specimens good electron microscope images can be

obtained and examined over prolonged periods. Moreover, the microscope vacuum is not compromised and potentially contaminating volatiles are avoided. Figures 2 to 4 show specimens which are typical of this class of material. Figure 2 is of wax from diesel fuel and Figure 3 is of asphaltene-rich deposits from crude oil. Both are beam sensitive at room temperature and both contain occluded solvents which, at room temperature, would volatilize in vacuo. Figure 4 shows the fat crystals which develop as a "bloom" on the surface of chocolate. At low temperature their form has been maintained despite prolonged exposure to the electron beam.

Specimens remain Fully Hydrated

At room temperature hydrated specimens rapidly lose water when subjected to the vacuum of the microscope. It is therefore common for most biological tissues, when examined "fresh" in this way, to shrink and distort even before the beam is switched on. The traditional solution to this problem has been to freeze-dry (FD) or critical-point-dry (CPD) such specimens before examination, usually after a preliminary fixation in chemical agents. Both drying methods were adopted to reduce specimen distortion associated with air drying but, as outlined below, other induced artefacts make these methods unacceptable for the preparation of many materials. Cryo-preservation of hydrated specimens facilitates their examination in the scanning electron microscope while constituent water is retained in a solid

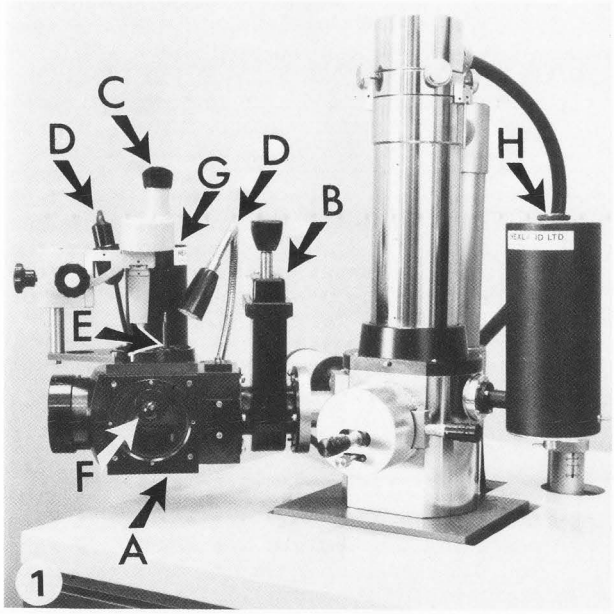

Figure 1. The Hexland CT 1000A Cryotrans system attached to a scanning electron microscope column. A: Preparation chamber. B: Gate valve. C: Binocular light microscope. D: Lamps. E: Sputter head. F: Freeze-fracture device. The preparation chamber stage is cooled by liquid nitrogen in Dewar G. Dry nitrogen gas circulating through the microscope stage is cooled by liquid nitrogen held in Dewar H.

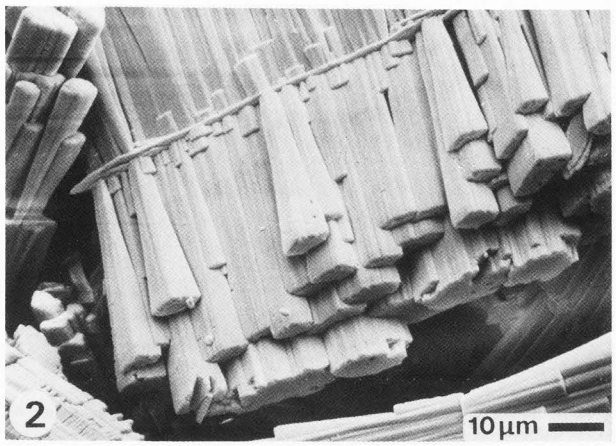

Figure 2. Wax deposits filtered from diesel fuel.

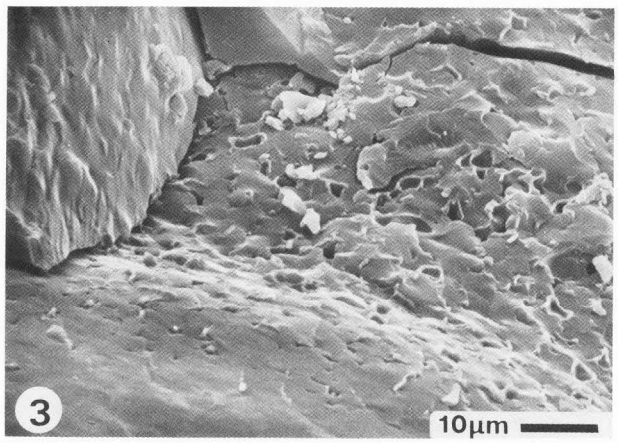

Figure 3. Freeze-fracture faces of an asphaltene-rich deposit from crude oil.

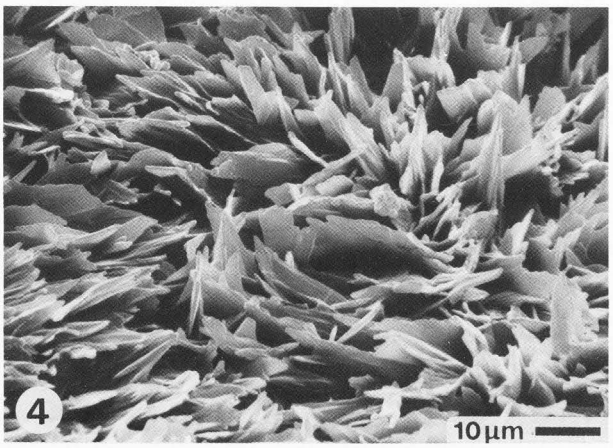

Figure 4. Chocolate "bloom". These crystals of fat (polymorphic form VI) develop on the surface.

form at a temperature too low even to permit sublimation. Water vapour pressure is virtually zero at temperatures below -130°C. Thus at -180°C, the cold stage operating temperature, hydrated specimens remain stable and available for prolonged examination.

Root hairs are ideal test subjects for a cryo-transfer system (Sargent, 1986a). They develop on most elongating roots as extensions of certain epidermal cells. Being highly hydrated and surrounded by only very thin cell walls they quickly collapse on losing water. Figure 5 is a micrograph of developing root hairs on cress, Lepidium sativum, prepared by cryo-preservation. Mucigel which is secreted by the root cap and the distal epidermal cells has been well preserved on the root surface. If such secretions are dried by whatever means, they usually adopt a lace-like appearance. Figure 6 similarly shows secretory tissue of animal origin. It is from the tongue of rat. The papillae which protrude from the surface carry, over at least their bases, a covering of mucus. At higher magnification and after light etching (Figure 7) this oral mucus can be shown to support a population of bacteria. Figure 8 shows the hydrated oral mucosa of the rat gum. The cell boundaries are clear and the technique retains those cells which are all but shed from the tissue. The squamous epithelium of the skin of guinea pig is shown in Figure 9. Although this tissue is not highly hydrated the micrograph illustrates well the advantage of cryo-preservation in retaining loosely held cells and remnants in situ. Another highly hydrated tissue is shown in Figure 10. It is the abscission zone at the base of the leaf of bean, Phaseolus vulgaris. Cells in this zone enlarge in response to ethylene and at leaf fall round off and separate from their neighbours through loss of cell-to-cell adhesion. Leaf shedding is the inevitable consequence. Through cryo-preservation the form and surface detail of these fragile cells has been perfectly preserved. Most fungal hyphae are very delicate and readily distort through water loss. Any study of host-parasite relationships requires that neither organism is displaced in relation to the other. Cryo-preservation maintains that association and both host and parasite remain fully hydrated. Figure 11 shows the powdery mildew Erysiphe galeopsides attached to the leaf of its host, red deadnettle.

Chemical Fixation is Avoided

Chemical fixatives have traditionally been used to stabilize tissues prior to dehydration. Rapid freezing obviates their use. By their nature fixatives are toxic to biological systems and present a hazard to users as do many buffers such as those containing cacodylate. Any procedure which circumvents their use is to be welcomed.

Specimens are not Immersed in Solvents

CPD was eagerly adopted by biologists as a convenient method for drying hydrated materials free from the distortion associated with the movement of a gas/liquid interface through the sample during air drying. Unfortunately, for many materials the artefacts induced by this method far outweigh those it was intended to avoid. CPD commonly involves fixation in one or more aqueous solutions, dehydration through a series of alcohol concentrations culminating in several transfers through absolute alcohol, exchange of the alcohol for acetone and exchange of the acetone for liquid carbon dioxide under pressure. Thus constituent parts of the specimen which are soluble in any of those solvents will be lost. The outer surfaces of most plants and many animals carry waxy structures which are readily lost during CPD (Sargent, 1983). Figures 12 and 13 show the surface of the broccoli leaf prepared by CPD and cryo-preservation, respectively. The natural "bloom" which is retained perfectly by cryo-preservation has been lose during CPD. The Whitefly, Aleyrodes brassicae (Figure 14) owes its colour to a dense layer of waxy particles which cover virtually the whole body except the compound eye and joints of the appendages. These particles are readily dislodged during specimen manipulation and dissolve in the solvents used during CPD. Cryo-preservation retains them intact (Sargent, 1986b). As Aleyrodes lays its eggs some of the maternal wax is transfered to their surfaces (Figure 15) but of particular interest is the annulus of insect wax which surrounds the point at which the end of the egg case is pressed into the food plant (Figure 16). On hatching, the larva supports little or no epicuticular wax (Figure 17) but during development a fringe of wax is secreted from its periphery (Figure 18).

Cryo-preservation is ideal for the observation of droplet and deposit distribution on surfaces following the application of pesticides (Hart & Young, 1987). Figure 19 shows the surface of a cotton leaf cryo-preserved within a few seconds of being sprayed with a pesticide formulation. Virtually no evaporation from the droplets had occured and the micrograph shows their size and distribution well. The technique can further be used to asses the deposition of non-volatile spray components following a dry-down period. Figure 20 is a micrograph of a wheat leaf cryo-preserved soon after a sprayed formulation had dried onto its surface. Not only is the pattern of solute aggregation clear but the effect of formulation additives in degrading the form of the leaf's natural wax "bloom" is apparent.

Volume Changes are Minimal

It is not uncommon for tissue shrinkage to reach 40% as a result of FD (Boyde & Franc, 1981; Boyde & Maconnachie, 1979) and CPD can also cause unacceptable dimensional changes (Beckett et al, 1984). Figure 21 shows the surface of a broccoli leaf after FD. Unlike the CPD-treated leaf the epicuticular wax has not been removed but its form has been dramatically altered (cf Figures 12 and 13). This disorganization results from shrinkage and distortion of the underlying epidermal cells which support the wax structures. Volume changes which accompany the cooling and freezing of water are well known but when compared to the dramatic effects that both FD and CPD have on tissue volume and form, they are minimal and for most purposes can be ignored.

Internal Structure can be revealed by Freeze-fracture

After due consideration is given to the possibility of internal ice crystal growth damage, much information can be obtained from an

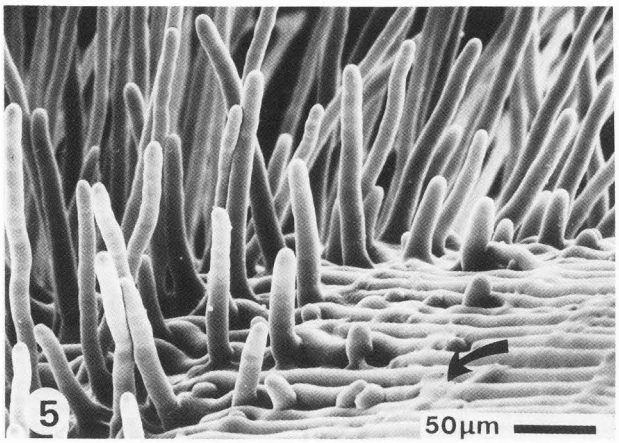

Figure 5. Developing root hairs of Lepidium sativum. These absorbing structures are extensions of certain root epidermal cells. Mucigel (arrowed) is well preserved on the root surface.

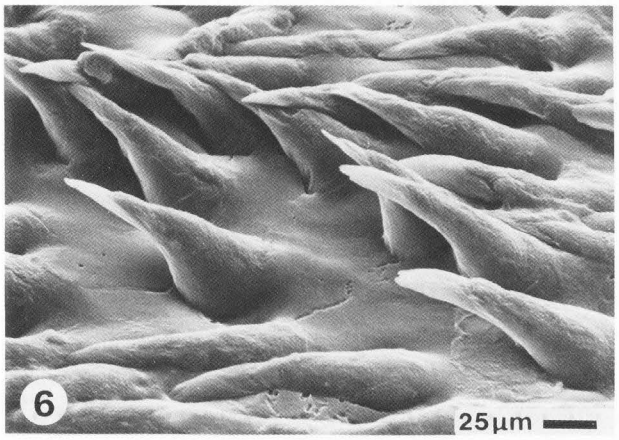

Figure 6. The surface of the tongue of rat. The papillae protrude through a layer of mucus.

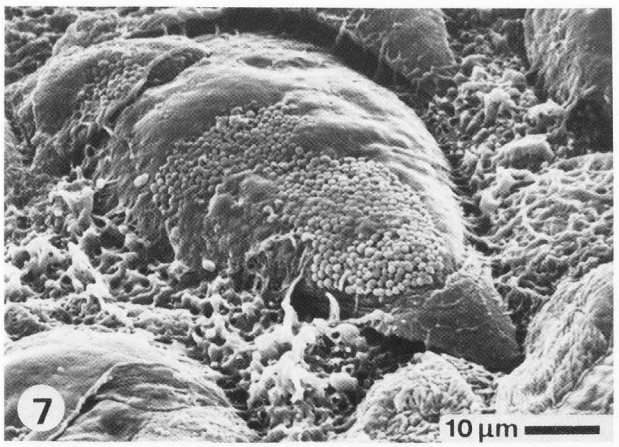

Figure 7. A preparation similar to that shown in Figure 6 which has been lightly etched to reveal bacterial colonies within the mucus.

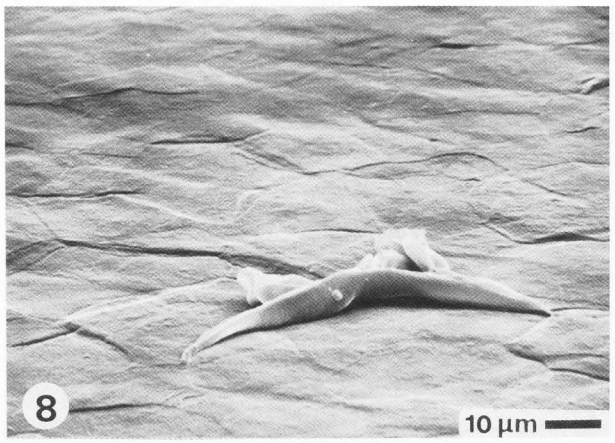

Figure 8. Oral mucosa from the gum of rat. Epidermal cells are continually shed from this tissue.

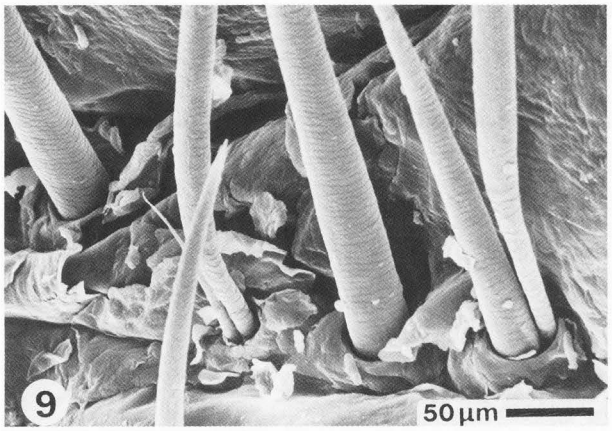

Figure 9. The skin of guinea pig. Shed cells and fragments from this squamous epithelium have been retained.

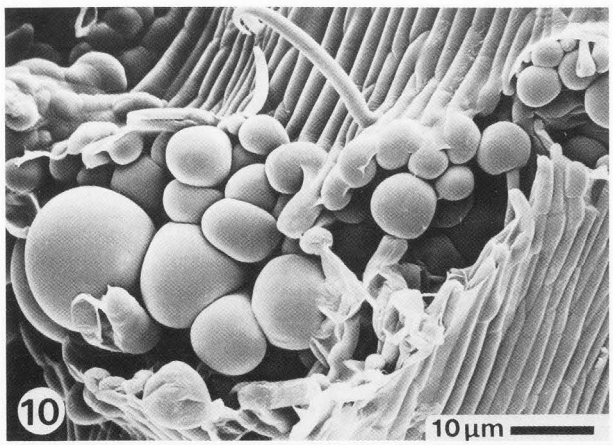

Figure 10. Enlarging cells of the abscission zone of Phaseolus vulgaris.

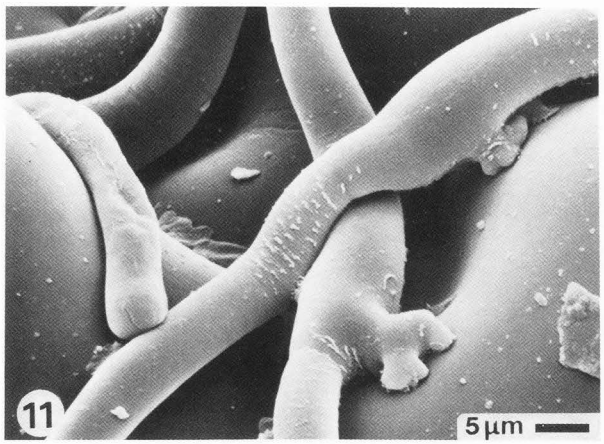

Figure 11. Hyphae of Erysiphe galeopsides on the surface of a red deadnettle leaf.

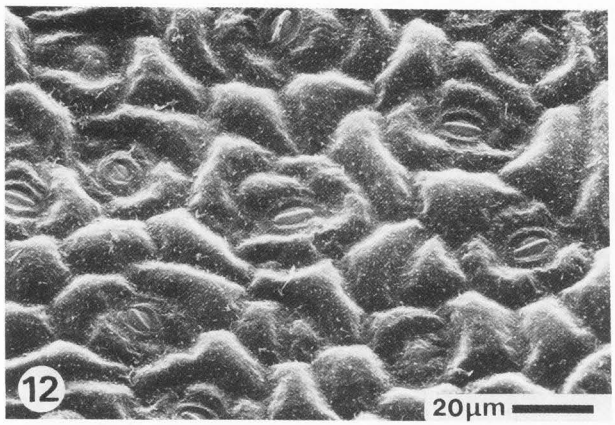

Figure 12. The surface of a broccoli leaf after critical point drying. Epicuticular wax has been dissolved.

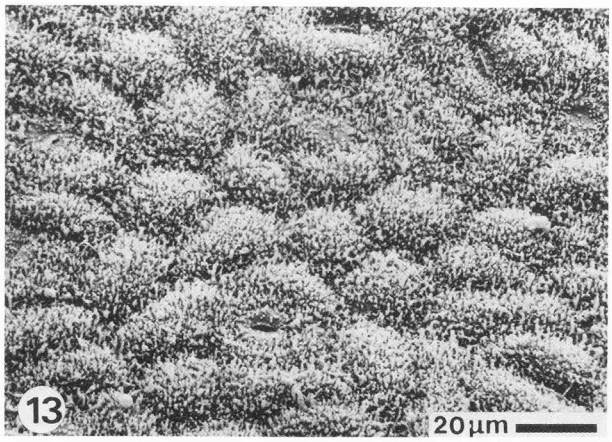

Figure 13. The surface of a cryo-preserved broccoli leaf. Epicuticular wax structures protrude from the surfaces of well defined, turgid underlying epidermal cells.

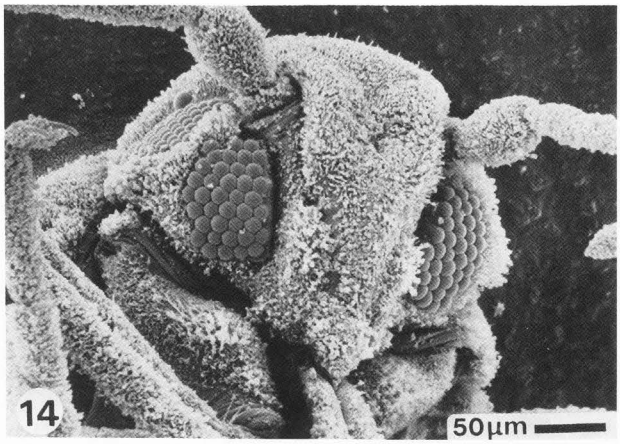

Figure 14. The head of Aleyrodes brassicae. Wax is secreted from almost the entire body surface. The eyes and appendage joints are exceptions.

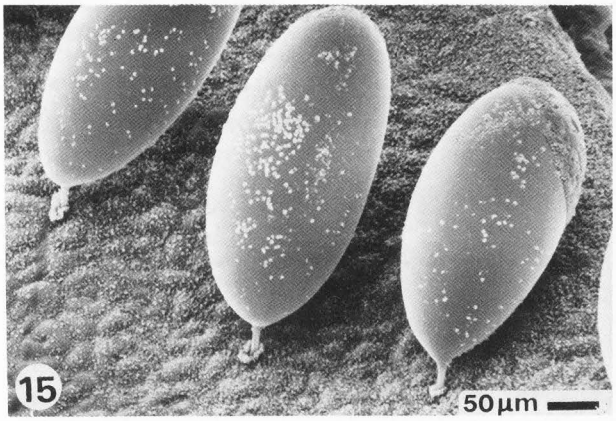

Figure 15. Freshly laid eggs of Aleyrodes brassicae. Their pointed ends are pushed into the surface of a cabbage leaf. Some maternal wax and other deposits are carried on them.

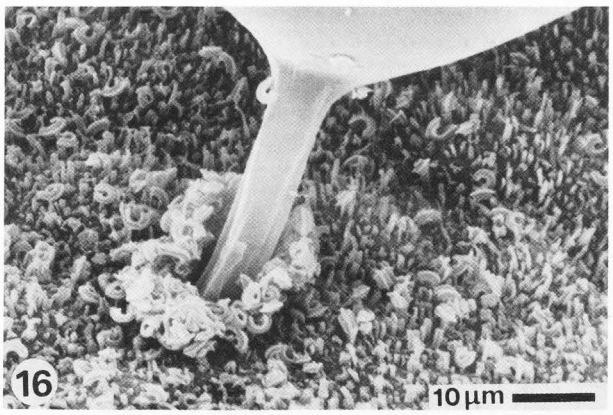

Figure 16. The base of an egg shown in Figure 15. A ring of maternal wax particles surrounds the point of insertion of the egg into the leaf. Maternal wax is scattered lightly over more remote areas of the leaf but its crescentric form distinguishes it from the straight wax rods which arise as a bloom on the leaf surface.

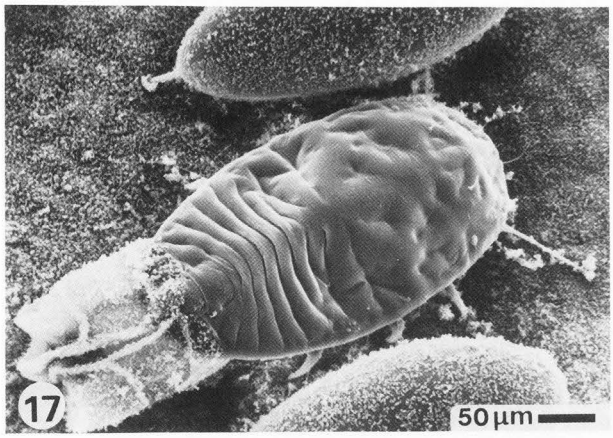

Figure 17. A larva of Aleyrodes brassicae emerging from its egg case. Its surface is virtually free of wax particles.

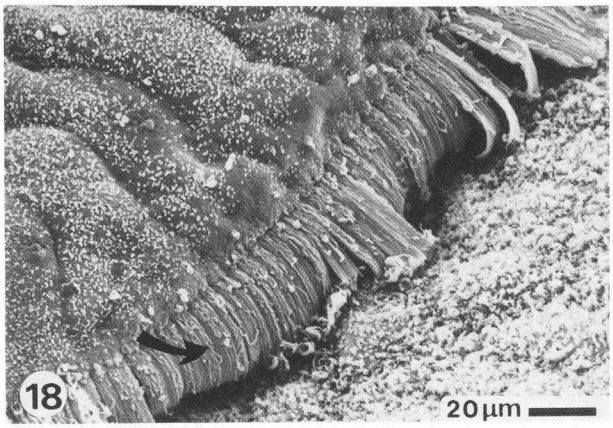

Figure 18. A peripheral fringe of wax (arrowed) is extruded from the edge of the developing larva of Aleyrodes brassicae.

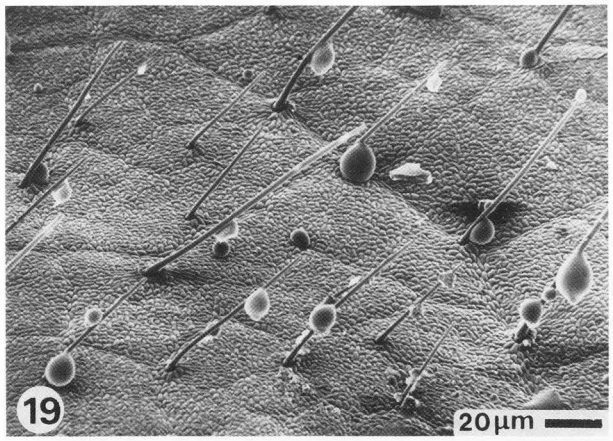

Figure 19. The surface of a leaf of cotton cryo-preserved immediately after spraying with an aqueous formulation of pesticide.

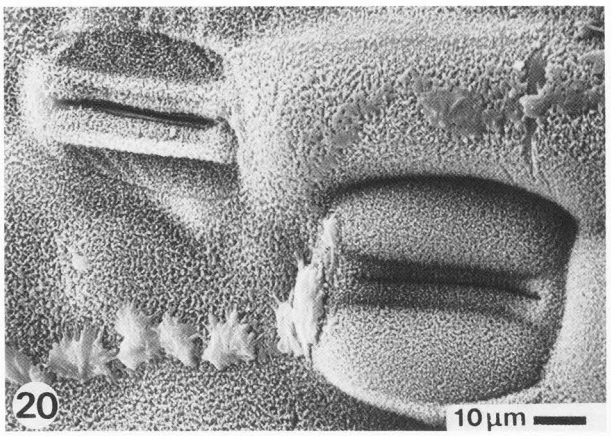

Figure 20. The surface of a wheat leaf cryo-preserved after the dry-down of a sprayed pesticide formulation. The form and location of the residue is apparent and effects of the formulation on the leaf's natural wax bloom can be observed.

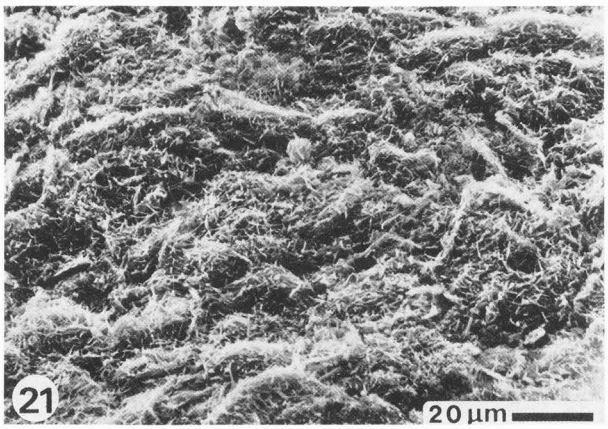

Figure 21. The surface of a broccoli leaf after freeze-drying. Shrinkage and distortion of underlying epidermal cells have disoriented the epicuticular wax particles.

examination of freeze-fracture faces. Ice cream is a perfect example with which to demonstrate the value of the technique. It is an oil-in-water emulsion in which are suspended air bubbles. The stability of this 3-phase system depends upon its enhanced viscosity at sub zero temperatures. However, the storage temperatures of this product are seldom low enough to prevent the slow growth of ice crystals and the development of graininess. By freeze-fracturing ice cream samples, parameters such as air bubble size and distribution (Figure 22) and ice crystal size (Figure 23) can be readily estimated. Most pharmaceutical creams are emulsified products containing 2 or more phases (Figure 24). The freeze-fracture technique likewise permits the rapid monitoring of droplet size and the degree to which liquid crystal formation has occured. The onset of component

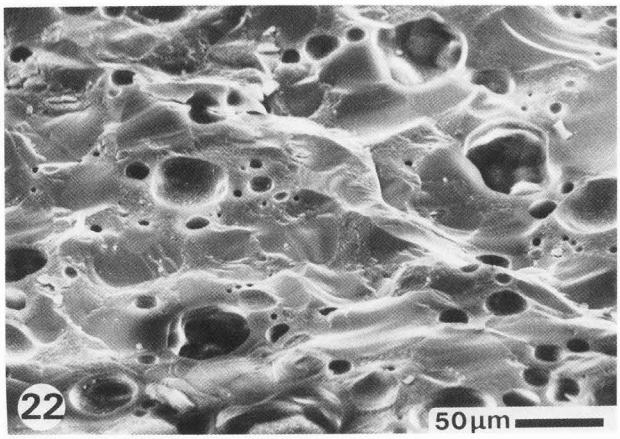

Figure 22. A freeze-fracture face of stored ice cream. The size and distribution of air bubbles can be assessed.

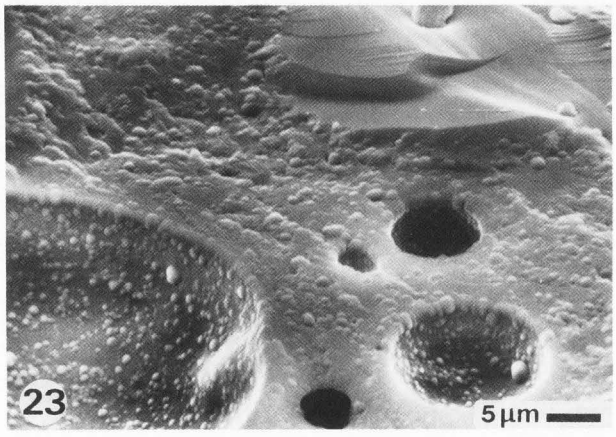

Figure 23. An enlarged area of Figure 22. The emulsified fat droplets are shown and the size of a developing ice crystal can be estimated from the area of its conchoidally fractured face.

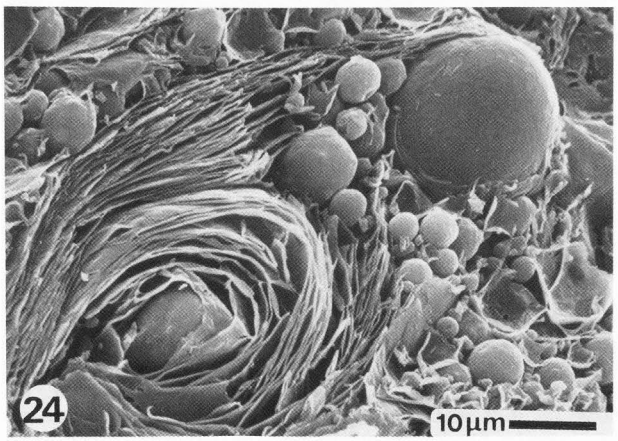

Figure 24. A pharmaceutical cream freeze-fractured and etched.

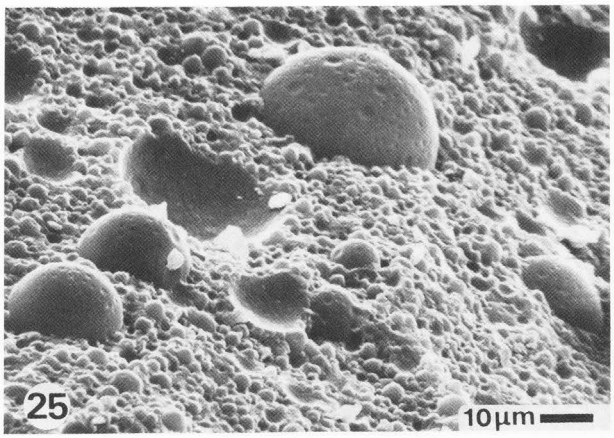

Figure 25. A freeze-fractured preparation of printer's ink. Coalescence of the suspended phase to form large droplets is indicative of the onset of phase separation.

separation through droplet coalescence is a problem encountered with many emulsified products. A freeze-fracture face of a sample of printer's ink is shown in Figure 25. The wide range of droplet size and particularly the presence of relatively large spheres suggests that in this sample coalescence is well advanced and that component separation is imminent.

The freeze-fracture technique is ideal for the internal examination of certain biological tissues. The anatomy of a walnut leaf is well illustrated in Figure 26 and the ramifications of the mycelium of Puccinia malvacearum within the leaf of mallow are shown in Figure 27. Figure 28 demonstrates not only the spatial relationship between pathogen and host in a rose leaf infected with Phragmidium but also the pectic strands remaining between the host cells after they had expanded and partially separated from one another during development. Two tissue types within the germinating grain of malting barley are shown in Figures 29 and 30. Figure 29 is a fracture through the scutellum and demonstrates the elongated form of the epithelial cells. In Figure 30 the fracture has passed between some cells of the aleurone, a layer of cells which secretes hydrolysing enzymes in response to a hormone from the embryo. The high accelerating voltage used in the preparation of this micrograph reveals the presence of granular components, the aleurone grains, beneath the cell surface. Figure 31 is of a freeze-fracture through the single-cell-thick aleurone layer of oat. The cells themselves have been fractured and light etching has exposed the aleurone grains as well as the nucleus within each cell.

The many tissues present within the ear lobe of mouse are revealed by freeze-fracture (Figure 32). Both sides of the ear bear hairs which arise through the surface epithelium from sub-epidermal follicles. Beneath these can be identified layers of connective tissue, vessels, muscle and cartilage.

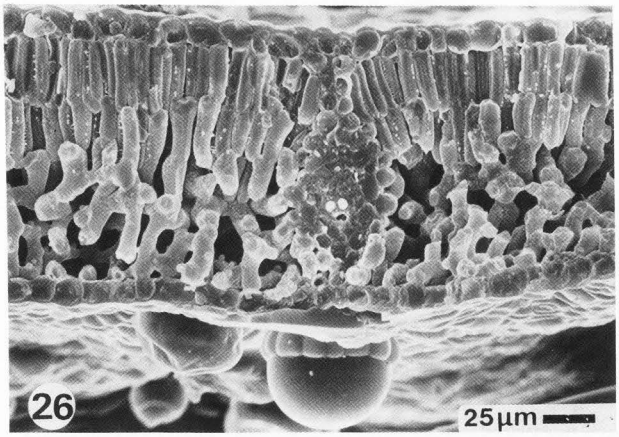

Figure 26. A freeze-fractured leaf of walnut. Columnar palisade cells beneath the upper epidermis overlie the spongy mesophyll. A vascular strand is shown in profile and epicuticular glands are evident on the abaxial surface.

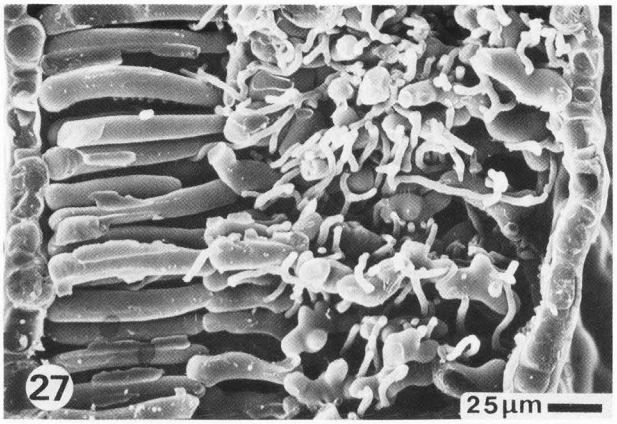

Figure 27. A leaf of hollyhock freeze-fractured to reveal the ramifying hyphae of Puccinia malvacearum with which it is infected.

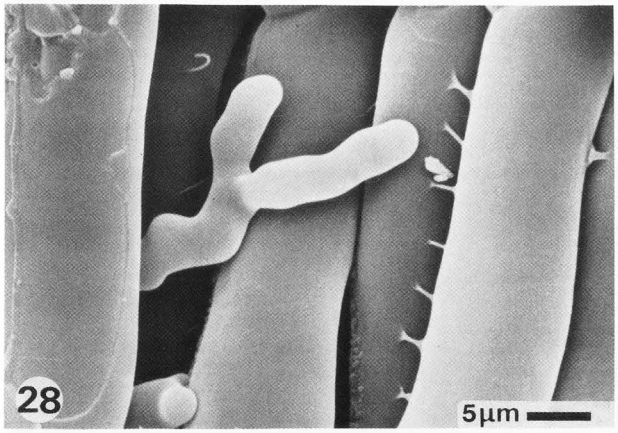

Figure 28. A freeze-fractured leaf of rose infected with Phragmidium mucronatum. Both the invading hyphae of the parasite and the intercellular pectic strands of the host are well preserved.

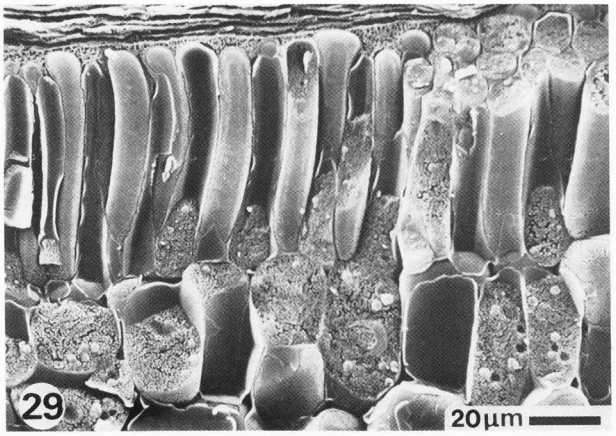

Figure 29. A germinating barley grain freeze-fractured through the elongated epithelial cells of the scutellum. Light etching has revealed some of the organelles within fractured cells.

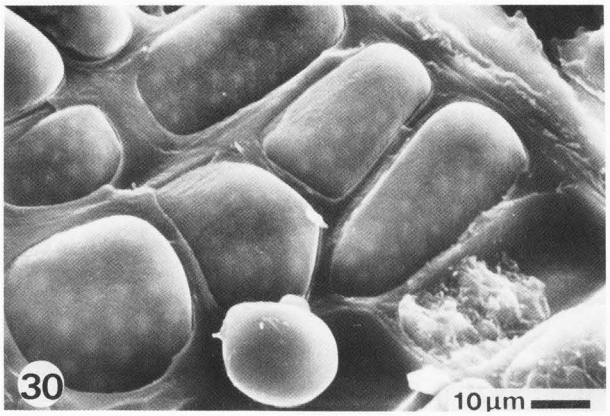

Figure 30. A germinating barley grain freeze-fractured between the plasmalemmae and walls of cells of the aleurone layer, Using an accelerating voltage of 25kV the position of underlying aleurone grains has been revealed.

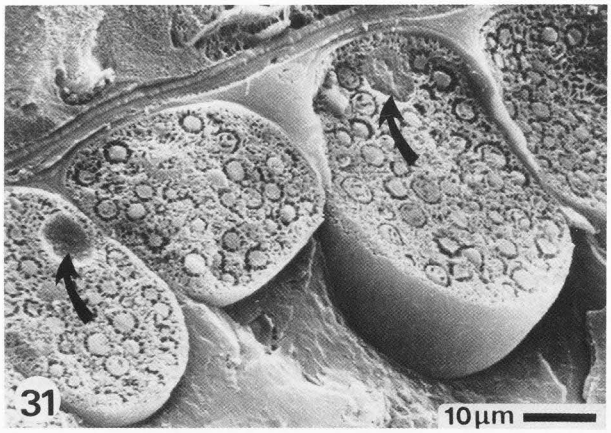

Figure 31. An imbibed seed of wild oat freeze-fractured through aleurone cells and lightly etched. The distribution of aleurone grains and the position of nuclei (arrowed) are revealed by this technique.

Elements are not lost or removed prior to X-ray Analysis

In preparing samples for X-ray microanalysis in the electron microscope, it is crucial to minimize the possibility of elemental translocation or leaching. This is impossible to guarantee, particularly for soluble components, if preparation involves chemical fixation or immersion in solvents. Cryo-preservation overcomes these problems. Rapid cooling and maintenance at temperatures far below those which permit movement of ions ensures that elements of interest are immobilized. The growth of ice crystals during specimen preparation can displace small particles and solutes to crystal boundaries but such events are unlikely to shift elements from one cell compartment to another. Provided no

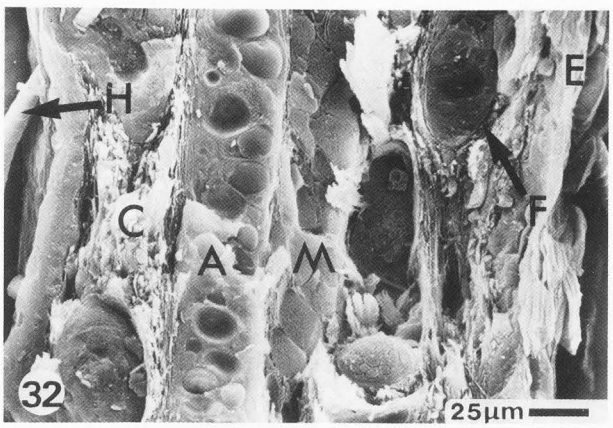

Figure 32. A freeze-fracture face across the ear lobe of mouse. Hairs (H), epithelial cells (E), hair follicles (F), connective tissue (C), muscle (M) and cartilage (A) are readily distinguishable.

Figure 33 to 36. Early events in the life of a larva of Pieris brassicae.

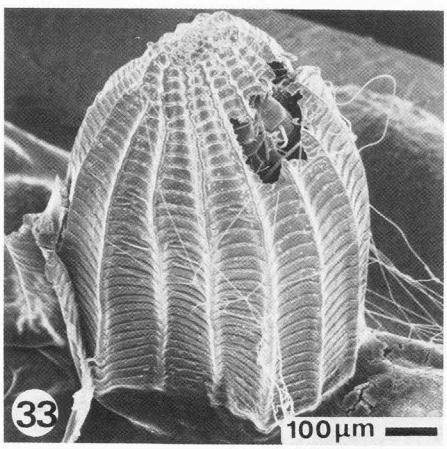

Figure 33. A larva cuts its way out of its egg case.

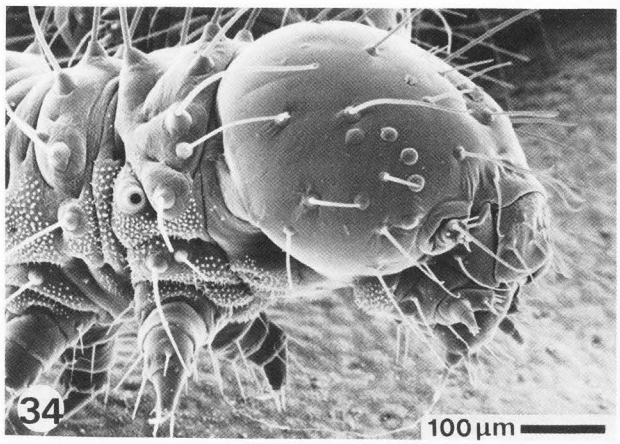

Figure 34. A larva spins silk.

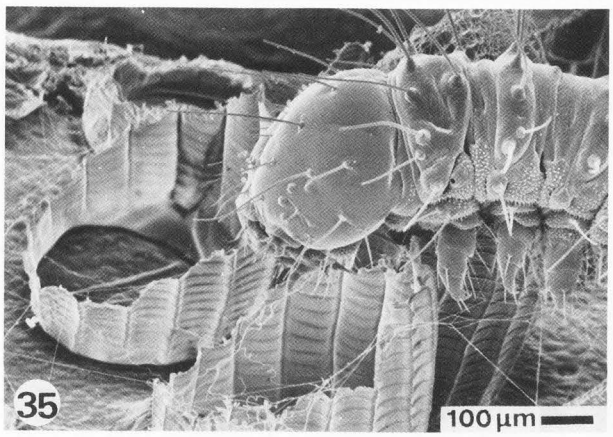

Figure 35. A larva consumes its egg case.

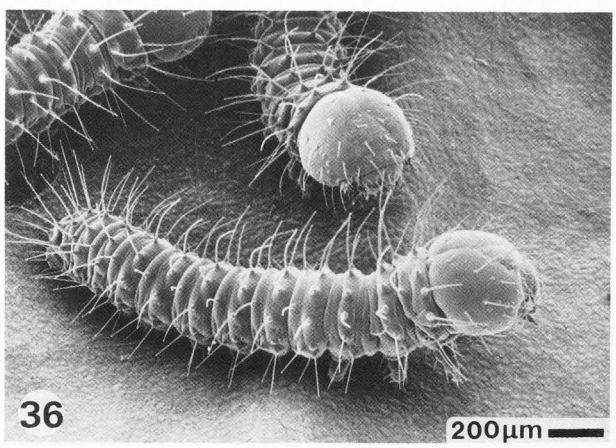

Figure 36. Larvae move to the edge of the leaf on which they were hatched to commence their vegetarian diet.

attempt is made to determine elemental distribution to a degree of resolution better than the dimensions of the ice crystals the technique may be used with confidence (Echlin et al,1982; Echlin & Taylor, 1986; Goldstein et al, 1981; Pitman et al, 1981). Ideally, specimens should be flat to obtain reliable quantitative data on element distribution. Surface roughness can be accomodated, within certain limits, using the peak to background ratio method (Echlin & Taylor, 1986; Marshall, 1980, 1984). This method cannot compensate for the gross irregularities in surface topography which are so often a feature of simple freeze-fracturing devices. To avoid such

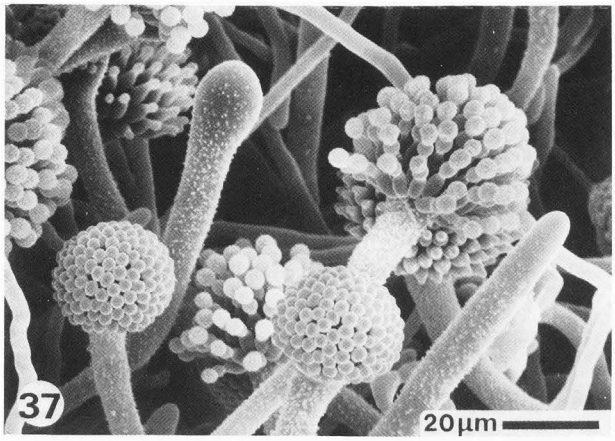

Figure 37. Developing conidiophores of the mould Aspergillus. The speed with which cryo-preserved specimens can be prepared enables a rapid search of material such as this to be made in order to select particular areas of interest.

Figures 38 to 45. Aquatic organisms collected on membrane filters and examined fully hydrated. Before freezing the specimens were washed well with distilled water and any excess water removed by sublimation on the microscope stage.

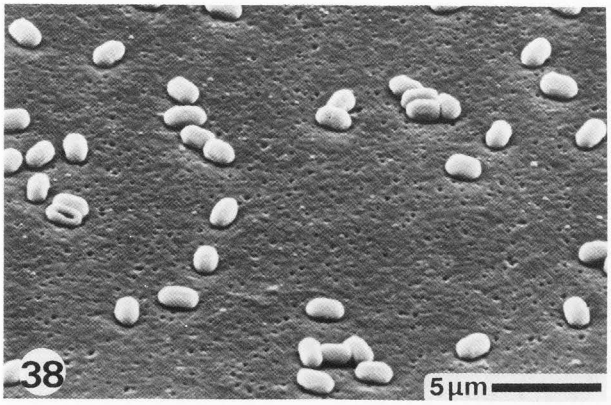

Figure 38. The bacterium, Bacillus subtilis var. globigii.

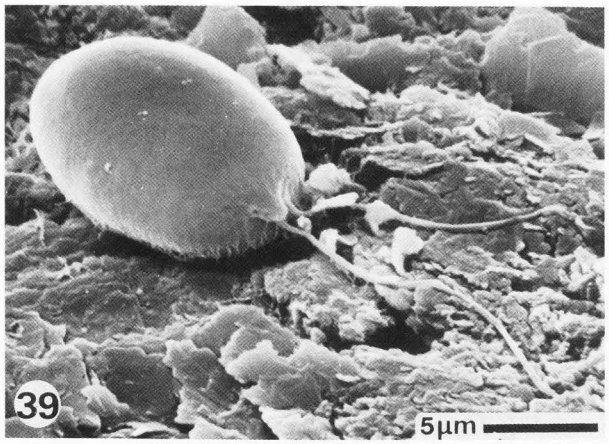

Figure 39. Chlamydomonas, a green unicellular alga.

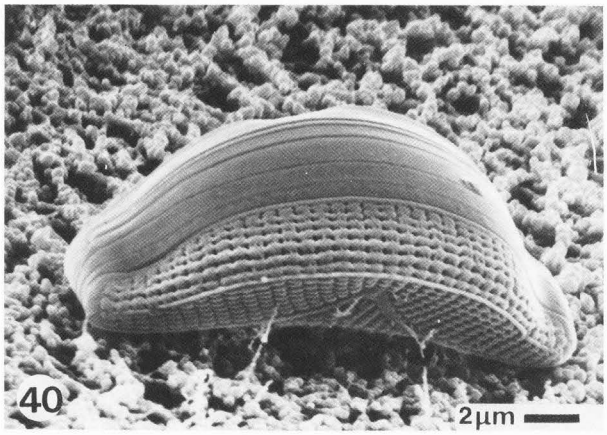

Figure 40. A unicellular diatom, Cymbella.

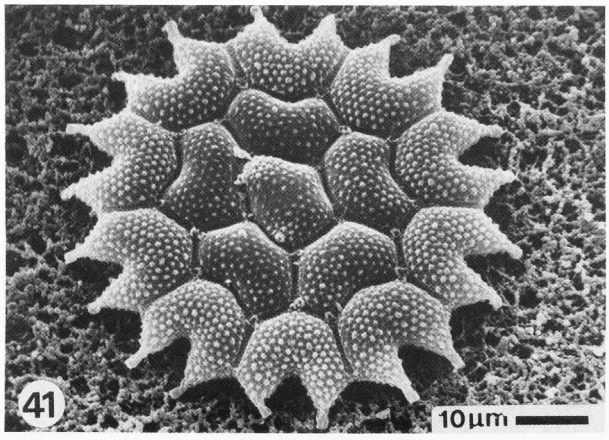

Figure 41. A colonial green alga, Pediastrum.

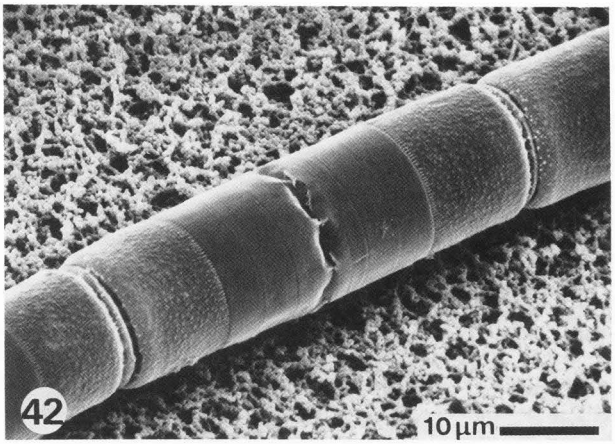

Figure 42. Melosira, a filamentous diatom.

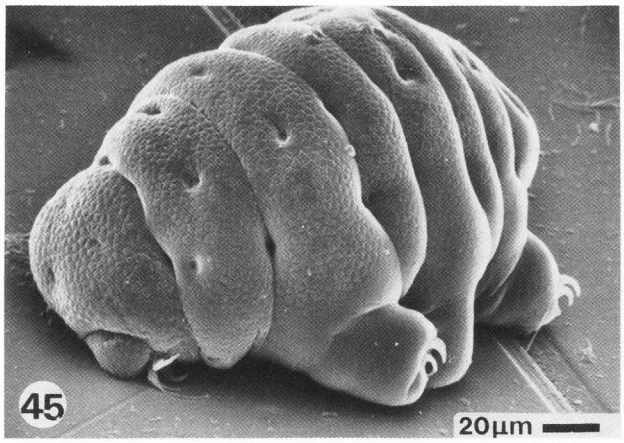

Figure 45. A tardigrade, Hypsibius oberhaeuseri.

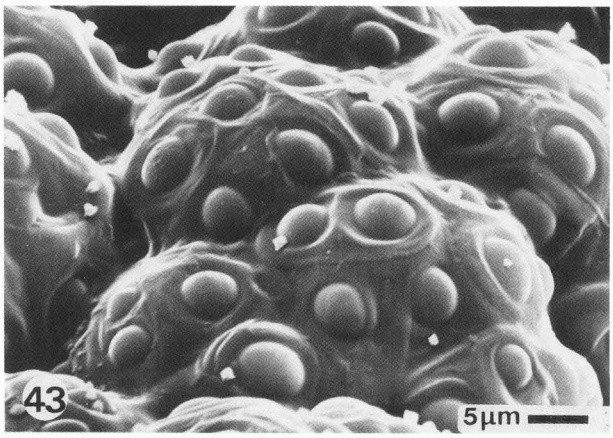

Figure 43. A colonial alga, Botryococcus, embedded within its buoyant matrix.

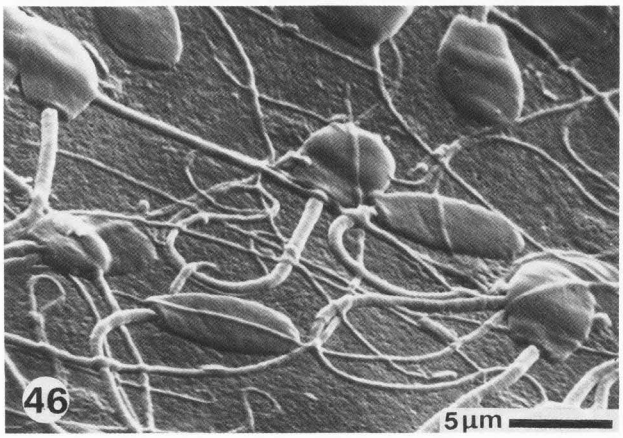

Figure 46. Spermatozoa of ram.

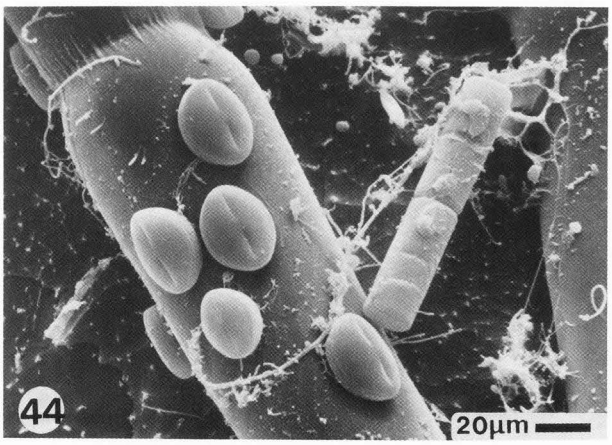

Figure 44. Diatoms, Cocconeis, epiphytic upon the filamentous alga, Cladophora.

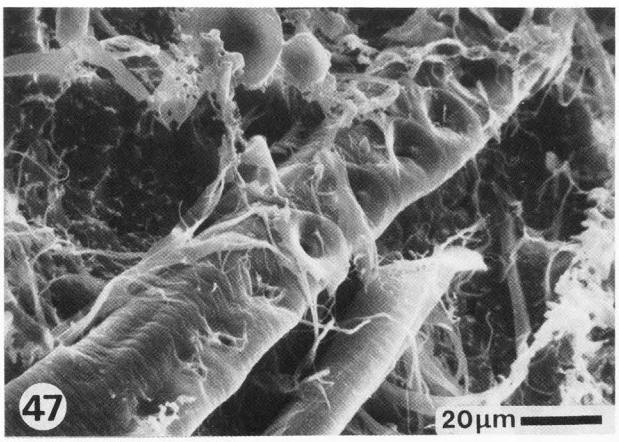

Figure 47. Wood pulp fibre.

irregularities the specimen can be cryo-honed. This is achieved in the CT 1000A using a cooled sliding knife which shaves the surface of the specimen by a series of cuts whose depth can be adjusted with a micrometer setting.

Motile Specimens are Arrested

An often overlooked advantage of cryo-preservation is the opportunity it offers to immobilize motile specimens instantly without recourse to anaesthetics or chemical fixatives. Sequences in a series of moving events can be arrested at intervals and held in a time-frame for examination at leisure in the scanning electron microscope. The series of micrographs in Figures 33 to 36 illustrate this. They are of young larvae of the large white butterfly, Pieris brassicae. Figure 33 shows a larva cutting its way out of its egg case, in Figure 34 a newly emerged caterpillar is spinning silk, Figure 35 shows one consuming its egg case and those in Figure 36 are moving away to find their first green meal.

Preparation is Complete within a few Minutes

A particular advantage of cryo-preparation is the speed with which it can be completed. The way in which many laboratories are now exploiting this has already been referred to. Until the cold stage and the cryotransfer system became available, scanning electron microscopy was regarded very often as an expensive facility demanding a large investment in time for sample preparation (from several hours to some days). Not only has that time been reduced dramatically but the potential throughput of prepared material has been increased. Specimens can be assessed, uncoated, on the microscope monitor within 2 or 3 minutes of being cooled. If they warrant no further examination they can be rejected and replaced over equally short time periods. Even when the "ideal" specimen is selected only a few further minutes are necessary to sputter-coat it in the preparation chamber prior to detailed examination and photography.

One appeal of the scanning electron microscope to biologists is its use in examining developmental stages of small organisms as they grow and reach maturity. Such observations are more convincing and appealing if a sequence of this sort can be captured on one micrograph. The search for that "special" micrograph has frequently been abandoned in the past because of the unacceptable demand on time that it was likely to make. With the remarkable reduction in sample preparation time now possible through the low-temperature technique, "special" micrographs are now less likely to be the consequence of luck, more the product of good judgement. Figure 37 shows a sequence in the development of conidiophores in a culture of the mould Aspergillus. Not only is that sequence captured on one micrograph but every part of this delicate organism appears perfectly preserved.

Small Specimens from Aquatic Environments

Small specimens from aquatic environments present special problems. They need to be collected, sometimes concentrated, and processed to make them suitable for viewing in the scanning electron microscope. Processing by traditional methods frequently results in their loss or gross distortion to their form. Using cryo-preservation they can be prepared rapidly and with confidence. The method generally involves collecting the specimens on a small membrane filter, washing them free of solutes, mounting the filter on a specimen holder, cooling it, removing any excess water by sublimation, and sputtering with gold. Examples of specimens prepared in this way are shown in Figures 38 to 47. Bacillus subtilis (Figure 38) is a bacterium which is commonly used as a monitor of sterilization techniques in the food industry. Chlamydomonas (Figure 39) is a unicellular green alga; its flagellae are well preserved. Cymbella (Figure 40) is a diatom, Pediastrum (Figure 41) is a colonial green alga, Melosira (Figure 42)is a filamentous diatom and Botryococcus (Figure 43) is a colonial alga which secretes a surrounding oily matrix. Figure 44 shows epiphytic diatoms on a filament of the alga Cladophora and Figure 45 is a micrograph of the tardigrade Hysibius oberhaeuseri, a small aquatic invertebrate which can withstand desiccation for long periods. Most sperm cells are easily prepared by this method. Those in Figure 46 are from the ram. They are paddle shaped and only half a micron thick. Wood pulp particles (Figure 47) are no longer part of a living system but, like other specimens in suspension, can be filtered and their structural features related to the techniques employed in their production (Howard, 1987).

Summary

The aim of this paper has been to demonstrate the opportunities which cryo-preservation provides for the scanning electron microscopist and to illustrate the very wide range of specimen types to which the technique can be applied. Through its use virtually all the artefacts associated with traditional methods of specimen preparation can be avoided. It has opened up new and exciting possibilities for all scanning electron microscope users.

Acknowledgements

The author thanks Mrs Jill Webb, AFRC Institute of Grassland and Animal Production, Hurley, UK for permission to publish Figures 20 and 26. In addition the author is grateful to the following publishers for their consent to reproduce particular figures in this text. Blackwell Scientific Publications: Figures 12, 13, 21; Longman Group Ltd: Figures 14, 35, 37; San Francisco Press Inc: Figures 2, 8 , 11, 17, 31, 39, 41.

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Discussion with reviewers

Reviewer III: How do you know that there has been virtually no evaporation from the droplets in Figure 19? What was the droplet size when sprayed?

Author: The leaf had been mounted on the specimen holder before spraying and the period before spray application and freezing was less than 2 seconds. It would be interesting to make a series of preparations with increasing, precisely measured periods between spraying and freezing. By plotting droplet size against time the initial average droplet size could be determined by extrapolation.

Rewiewer III: What is the time sequence, in natural life, for the events illustrated in Figures 33 to 36? Why is cryo SEM necessary for this sequence? Could it not be done with careful CPD procedures? Was this sequence taken of different preparations from the same leaf on the same plant?

Author: I do not know the precise time sequence illustrated here. All these events were observed on 2 colonies of hatching and developing larvae. The course of these events could be followed precisely using a dissecting microscope but only cryo-preservation can arrest development and movement at once and, in addition, maintain the insect in a hydrated form for prolonged examination. Even if CPD was used the problem of immobilization would not be solved. Chemical fixation would not arrest movement in the way illustrated so dramatically in Figure 35. Neither would CPD be so simple nor so fast to perform.

Reviewer III: What are the effects induced by the growth of ice crystals deep within frozen-hydrated bulk material?

Author: When ice crystals grow they not only push aside other non-soluble structural components but they also displace solute molecules towards crystal boundaries. It is therefore important in the examination of freeze-fracture faces either to prevent ice crystals growing to a significant size or recognise the changes induced by their growth.

A.W. Robards: How effective is an anti-contaminator plate at -180°C when the specimen stage is at the same temperature? What is the actual specimen temperature?

Author: When both anticontaminator and stage are at the same temperature of -180°C no water can be lost from the specimen and none can be transfered from the anticontaminator to the specimen. The water vapour pressure at this temperature is zero. Only when the stage temperature is raised to sublime water (e.g. at -80°C is there water vapour in the specimen chamber. It will then condense on the cold anticontaminator, not the stage. To maintain the specimen temperature close to that of the stage it is important that it has good thermal contact with the stage. Bulk specimens should be firmly glued with a thin layer of heat conducting cement and fracture rivets should be firmly clamped to the specimen holder. The specimen temperature can rise significantly under the electron beam if the specimen itself is a poor conductor of heat. Under those circumstances it is vital to keep the stage at its lowest possible working temperature and to restrict the height of the specimen above the stage to a minimum.